JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2010. Vol. 4(1), p. 207-214

Use of Silver Nanoparticles from *Fusarium oxysporum* in Wound Dressings

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(Received: 10 September 2009; accepted: 25 November 2009)

Microbial resistance exacerbated by the overuse of antibacterials requires novel methods to manage topical infections. Inclusion of silver nanoparticles in medicinal dressings can serve as an alternative method to alleviate emergence and spread of antibiotic resistance. Biosynthesis of silver nanoparticles was carried out using *Fusarium oxysporum* and its presence was detected visually by appearance of brown color and using UV-Visible spectrophotometer wherein an absorbance maximum was observed at 420 nm. *Fusarium oxysporum* was found to accumulate about 0.4 mg of silver nanoparticles per gram of biomass with a size range of 20-25 nm as detected by SEM and Atomic absorption analysis. MIC of free silver nanoparticles was found to be within the range of 12 to 28 ppm for *Escherichia coli, Enterobacter cloacae, Pseudomonas aeruginosa* and *Proteus vulgaris.* Also no viable organisms of the above cultures were obtained after 4 hours indicating an effective bactericidal activity. In contrast *S. aureus* was found to be resistant to 34 ppm of silver nanoparticles and showed only 2 log cycle reduction within 4 hours indicating its resistant nature.

Key words: Silver nanoparticles, Fungi, Wound dressing.

Wounds act as an important focus for cross-infection, necessitating the application of appropriate measures to eliminate its spread. The strong inhibitory effects of silver towards a broad spectrum of bacterial strains in its different oxidation states (Ag^0, Ag^+, Ag^{2+}, and Ag^{3+}) along with silver nitrate and silver sulfadiazine have been known for a long time and silver has been traditionally accepted as an antimicrobial agent for the treatment of superficial, deep dermal burns and warts[1]. Thus incorporation of inorganic silver in coated dressings will form an effective means of treatment as it kills a broader range of bacteria than the cream base, is better adsorbed, and less irritating than the silver nitrate solution. Additionally a small sized molecule allows better penetration into cells and thus small sized fabricated nanoparticles of silver if incorporated onto materials used for wound management can effectively enhance its bactericidal activity with reduced irritability.

Many microorganisms have the ability to produce inorganic nanostructures and metal nanoparticles with properties similar to chemically-synthesized materials, while exercising control over the size, shape and composition of the particles. This provides an alternative approach to chemical synthesis procedures that uses microbial systems for the production of nanosized materials which can be cost effective as it enables harvest of a particle with a uniform well defined size. Additionally, the use of fungal cultures for production of such materials will have the added advantage in downstream processing besides being simpler and cost effective in its handling procedure. Thus this study aims to biosynthesize silver nanoparticles using fungal biomass for its effective use in wound dressing materials

The ability to synthesize silver nanoparticles has been reported in *Pseudomonas*

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stutzeri AG259[2], *Lactobacillus* strains[3], *Phoma*[4], *Verticillium*[5], *Fusarium oxysporum*[6], *Aspergillus fumigatus*[7], *Phaenerochaete chrysosporium*[8] and silver-tolerant yeast strain MKY3[9].

The study of antibacterial activity of silver nanoparticles has been reported by Pal et al where the antibacterial efficacy on *Escherichia coli* depended on the shape of nanoparticles, with truncated triangular particles showing almost complete inhibition at 1 ppm while rod-shaped nanoparticles and silver nitrate showed inferior performance with its minimum inhibitory concentration being more than 100 ppm. In contrast, spherical silver nanoparticles, above 12.5 ppm was required to reduce the growth significantly, though a total of 50 to 100 ppm of silver nanoparticles was required for 100% inhibition of bacterial growth[10].

Tian *et al* have reported that topical delivery of silver nanoparticles promoted wound healing in an in vivo model. In animals treated with silver nanoparticles healing took place within 26.5+0.93 days as compared to the normal 35.4+1.29 days. Wounds treated with silver sulfadiazine however took 37.4+3.43 days to heal[11].

In a study done by Duran et al 99.9% reduction of *Staphylococcus aureus* was observed with cotton fabrics impregnated with silver nanoparticles produced by *Fusarium oxysporum*[12].

The aim of the present study was to study the antibacterial effect of silver nanoparticle impregnated bandages on wound pathogens.

MATERIAL AND METHODS

Biomass production

5 days old *Fusarium oxysporum* (MTCC: 284) grown on PDA slant was inoculated in 500 ml Erlenmeyer flasks each containing MGYP medium (100 ml). The flasks were incubated at 25–28°C under shaker conditions (200 rpm) for 96 hours and mycelial mass thus obtained was separated from the culture broth by centrifugation (5000 rpm) at 10°C for 20 minutes. The mycelia was washed thrice with sterile distilled water and used for induction of silver nanoparticles synthesis. Additionally the fungal filtrate was also checked for its ability to induce biosynthesis of silver nanoparticles[6].

Biosynthesis of Silver Nanoparticles

20 g of fungal biomass was inoculated into 100 ml aqueous solution of 1 mM silver nitrate and incubated in dark under shaker conditions (200 rpm) for 96 hours at 28°C. The bio-transformation for production of silver nanoparticles was monitored by visual inspection with respect to color change as well as using UV-Visible spectrophotometer.

Visual inspection

Production of silver nanoparticles was detected through the change in color from pale yellow to brownish[6].

UV-Visible spectrophotometer analysis

Aliquots of the solution were removed and the absorption was measured in a UV-Visible spectrophotometer (UV Dec 40- double beam) wherein a peak within the range of 410 - 460nm characterizes the presence of silver nanoparticles[6].

Purification & quantification of the fungal silver nanoparticles

The reaction mixture showing a brown color and an absorbance peak between 410-460 nm after 96 hours was subjected to ultracentrifugation (Heraeus centrifuge-1) at 15000 rpm for 20-30 minutes. The isolated silver nanoparticles were washed with distilled water, centrifuged at 15000rpm for 20-30 minutes to obtain purified silver nanoparticles.

Quantification of silver nanoparticles was done using Atomic Absorption Spectrometer using Chemito AA 203 containing silver cathode lamp at a wavelength of 338.5 nm. Fresh solutions of $AgNO_3$ containing 1 mM, 2 mM and 3 mM were used for preparation of a standard curve. Absorption readings from the supernatant after ultracentrifugation were then compared to the standard curve for determination of residual silver concentration and quantified using Dias et al formula [4]:

Characterization of silver nanoparticles

SEM: Images of silver nanoparticles were obtained using a Hitachi 4500 scanning electron microscope (20KeV, 11.0K magnification) to determine the size & shape of the silver nanoparticles

Antibacterial activity of colloidal silver. MIC

MIC of silver nanoparticles against hospital strains of common burn wound pathogens (*S. aureus, P. aeruginosa, E. coli, E. cloacae,*

P. vulgaris) was determined by tube method. Cultures were grown in Nutrient broth supplemented with silver nanoparticles in the range of 0.15 ppm – 34 ppm and its inhibitory concentration was determined after incubation at 37^0C for 24 hours.

Killing efficacy

The killing efficacy of free silver nanoparticles against the above listed pathogens was determined by counting the viable bacteria in nutrient broth supplemented with minimum inhibitory concentration of silver nanoparticles after a period of 0,2,4 and 6 hours.

A suspension of pathogenic cultures was prepared from colonies grown on BHI agar. Bacterial suspension was then added to nutrient broth containing silver nanoparticles at its minimum inhibitory concentration. Control broths with and without bacterial inoculation were also included. The tubes were then incubated with agitation at 220 r.p.m. and aliquots were sampled from each vial at intervals of 0, 2, 4 and 6 hours. Serial dilutions for each aliquot were prepared and viable count performed by Miles & Misra technique[13].

Efficacy of silver nanoparticle impregnated wound dressings against burn wound pathogens.

Preparation of silver nanoparticle impregnated wound dressings

Bandage materials procured from Komal Healthcare Pvt. Ltd., Mumbai was incorporated with silver nanoparticles using paraffin base melted at 60^0C. Silver nanoparticles at concentrations were mixed with the paraffin base such that the final concentration was equivalent to the MIC determined earlier and poured over the sterilized mesh of cloth. Such silver nanoparticle incorporated dressing was then used to study its antimicrobial potential.

Efficacy of dressings impregnated with silver nanoparticles

Dressing were cut in squares of size 1cmX1cm and placed in a sterile vial. The dressing was then subjected to washing for 10 minutes using distilled water. Tryptone soy broth (5 ml) was then added to each vial. 5 ml suspension of 24 hours old bacterial culture was then added to each vial containing the dressing. Control broths with and without bacterial inoculation were also included. The tubes were then incubated on shaker. Aliquots of broth were sampled from each vial at time intervals of 0, 2, 4, 6 hours. Serial dilutions for each aliquot were prepared and viable count was performed by Miles & Misra technique [13].

RESULTS AND DISCUSSION

Screening of extracellular synthesis of Silver Nanoparticles

Visual Inspection

Capability of *Fusarium oxysporum* biomass to convert silver nitrate to silver nanoparticles was observed through a change where in an almost pale white to colourless solution changed to brown colour within 96 hrs . This is similar to the colour transformation reported by Senapati et al[6] for all their fungal cultures indicating the ability of our culture to initiate the silver biotransformation within 48 to 96 hrs.

UV-visible spectroscopy

Absorption spectroscopy in the UV-visible region has long been an important tool for the nanomaterial characterization. The atomic absorption spectra of nanoparticles are dominated by surface plasmon resonance (SPR) and their number and shifts to longer wavelengths indicates increasing particle size. Mie reported that a single SPR band is the expected absorbance spectra of spherical nanoparticles whereas anisotropic particles would give rise to two or more bands depending on the shape of the particles[10]. In our studies the absorption spectrum of the sample containing *Fusarium oxysporum* cells immersed in 1mM $AgNO_3$ showed a single surface plasmon absorption band with a maximum 420 nm indicating the lone presence of spherical or roughly spherical Ag nanoparticles. Duran et al reported the role of *Fusarium oxysporum s*pecific anthraquinones dependent reductase in the biotransformation process due to its ability to reduce Ag^+ ions that led to the formation of silver nanoparticles was mainly due to a conjugation between the electron shuttle with the reductase participation[14].

Though *Fusarium spps* are alike in the production of naphthaquinones they differ in the production of anthraquinones a characteristic not

(a) Pale white color observed immediately after exposure of fungal biomass to 1mM $AgNO_3$

(b) Color transition to brown color after 96 hrs.

Fig. 1. Detection of Ag nanoparticles by color transition

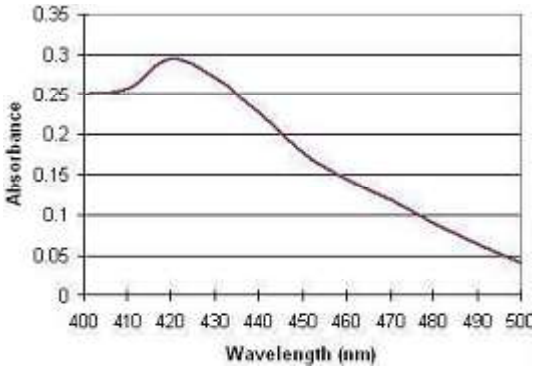

Fig. 2. Detection of Ag nanoparticles through UV-visible spectroscopy

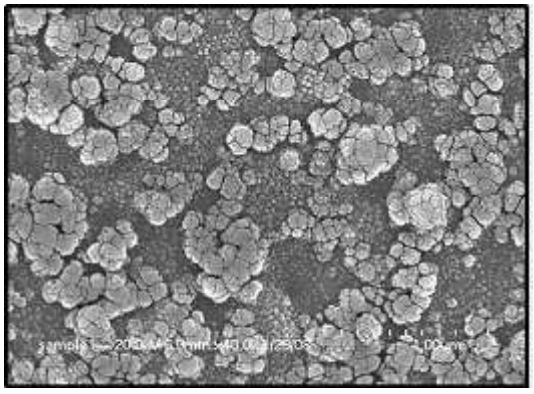

Fig. 3. SEM micrograph of Ag nanoparticles formed by *Fusarium oxysporum*

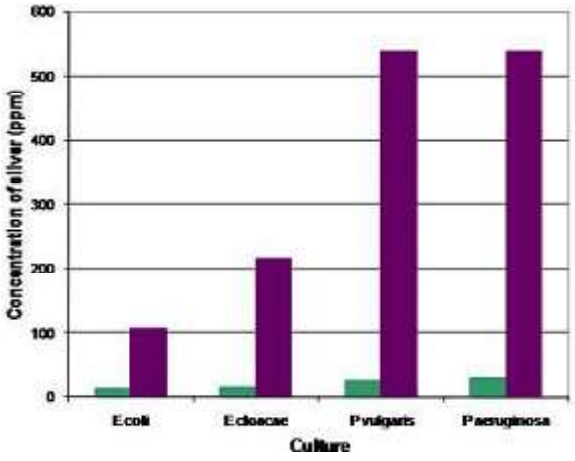

Fig. 4. MIC of Ag nanoparticles formed by Fusarium oxysporum on wound pathogens

detected in *Fusarium moniliforme*, indicating that this trait is not universal in the *Fusarium* genera[14].

Purification and quantification of silver nanoparticles

After ultracentrifugation at 15000 rpm, a brown color pellet of silver nanoparticles with a yellow color supernatant was obtained. Atomic Absorption Spectroscopy of the supernatant was carried out to quantify silver nanoparticle production. A bioconversion efficiency of 20% with a harvest of 0.4mg/gm fungal wet biomass was attained (Dias et al) as 100 ppm of silver nitrate yielded 19.81ppm of nanoparticles. A similar study using *Phoma* spps was done where in a yield of 13.4 mg of silver was obtained per gram of dry mycelium[4].

Characterization of silver nanoparticles

Scanning electron microscopy

SEM picture showed individual silver nanoparticles as well as a number of aggregates. Silver nanoparticles were found to be spherical in shape within the size range 20 nm. The silver nanoparticle solution synthesized by the reaction of Ag^+ ions with *Fusarium oxysporum* was exceptionally stable retaining its integrity for a period of 2-3 months due to the capping phenomenon with proteins secreted by the fungus that prevented an increase in size of the molecule with aging. This corroborates well with the observation [6, 14] which showed spherical nanoparticles within the size range 20–50 nm using *Fusarium oxysporum.*

Determination of Minimum inhibitory concentration of the silver nanoparticles

Minimum inhibitory concentration of silver nanoparticles obtained from *Fusarium oxysporum* was found to be lowest for *Escherichia coli* at 12ppm and within the range of 14ppm to 28 ppm for *Enterobacter cloacae*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. *Staphylococcus aureus* was found to be resistant to the silver nanoparticles up to a concentration of 34ppm indicating that silver has a better effect on Gram negative microorganisms than Gram positive microorganisms.

In a study done with *Escherichia coli* ATCC-15224, silver nanoparticles at concentrations of 60 ppm and higher were found to be effective bactericides [15]. Shrivastava et al reported that 25ppm of silver nanoparticle was found to be effective in inhibiting *Escherichia coli* along with a variety of other resistant or non-resistant strains of gram-negative bacteria. Similar to our results silver nanoparticles were found to have a less significant effect on the growth of *Staphylococcus aureus* with no reduction in bacterial growth observed till the concentration of nanoparticles increased to 25ppm and atleast a concentration of 100ppm was required for partial growth inhibition [16].

Comparison of the effectiveness of the formed silver nanoparticle with silver nitrate solution revealed ability of silver nitrate to inhibit the test culture within a range of 108ppm-540ppm of silver indicating that silver nanoparticles are much more effective than molecular silver or silver salts. This could be due to the high surface to volume ratio of the silver nanoparticles and the presence of active facets in different shapes of nanoparticles.

Killing efficacy of free silver nanoparticles

Though silver nanoparticles showed low MIC values against Gram negative pathogens, its killing efficacy as determined by the time required to attain at least 90% kill were assessed.

Silver nanoparticles at 34ppm concentration showed complete bactericidal activity within 2 hours for *Escherichia coli* whereas other gram negative cultures were killed within 4 hours. *Staphylococcus aureus* showed only 2 log cycles reduction in 6 hours indicating it may be ineffective against Gram positive organisms.

Effective killing rate of the produced nanoparticles from *Fusarium oxysporum* thus can enable its use as germicidal bandage. Thus silver nanoparticles were impregnated onto dressing material at a concentration of 34ppm per cm^2.

Effect of different bandages/dressings on pathogens

A rapid bactericidal action would permit wound healing to proceed without bacterial interference and reduces the likelihood for resistance to develop. Thus the bactericidal activities of the silver nanoparticles impregnated dressing (3.4mg/100cm^2) against five bacteria were studied.

Silver nanoparticle impregnated dressing was found to be effective especially on Gram negative microorganisms such that within 6 hours

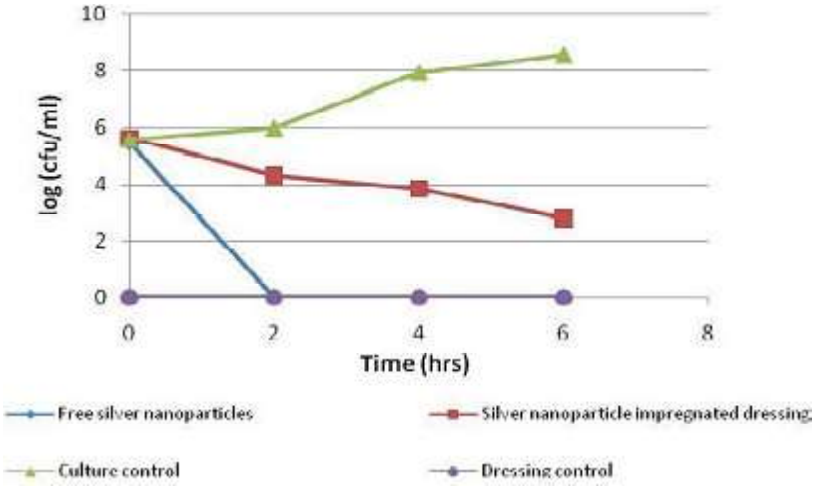

Fig. 5(a). Effect of free Ag nanoparticles and silver impregnated dressings on *Escherichia coli*

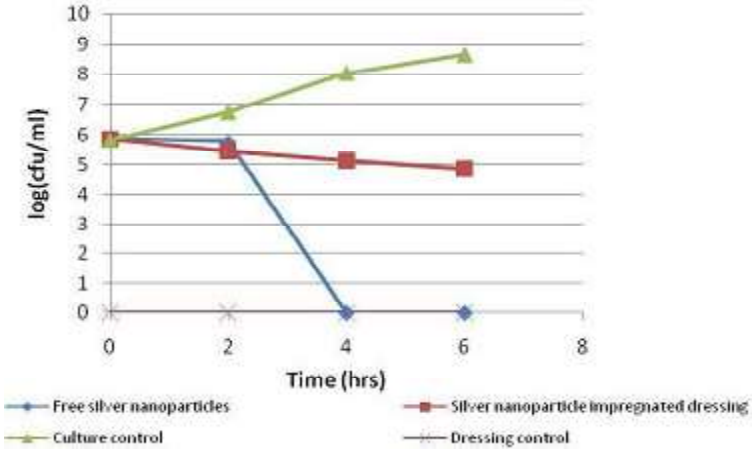

Fig. 5(b). Effect of free Ag nanoparticles and silver impregnated dressings on Enterobacter cloacae

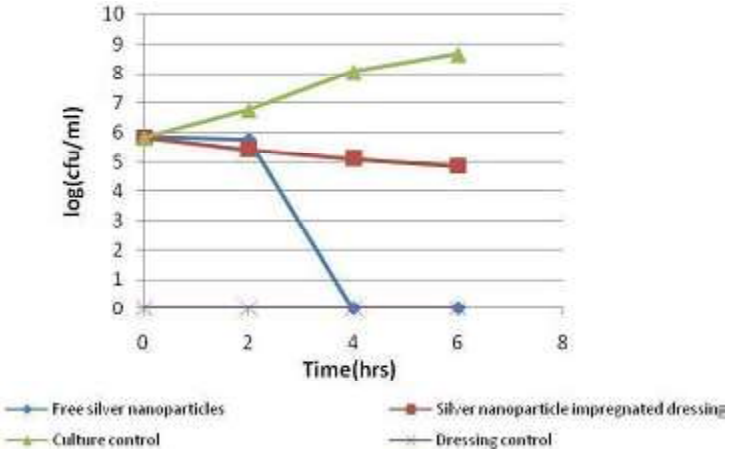

Fig. 5(c). Effect of free Ag nanoparticles and silver impregnated dressings on Proteus vulgaris

three log cycles reduction were observed in case of *Escherichia coli* and *Pseudomonas aeruginosa* . However two log cycle reduction was seen in *Proteus vulgaris* and one log cycle reduction was seen in case of *Enterobacter cloacae* within 6 hours. Such a bactericidal activity of free silver nanoparticles (t =3.269) and silver nanoparticle impregnated dressing (t = 2.416) against these organisms was found to be statistically significant though silver nanoparticle dressings did not show reduction in the bacterial counts till 6 hours for *Staphylococcus aureus*.

Margaret *et al*[13] have studied the comparative data of the antimicrobial efficacies of various silver dressings/bandages and have reported that all the silver-impregnated dressings were bactericidal on the coliforms and achieved >100000 c.f.u./ml reduction of *Escherichia coli*, *Proteus vulgaris* and *Enterobacter cloacae* between 30 min and 24 hours in contrast the action on Gram-positive bacteria which was least satisfactory, with bacterial growth not inhibited even after 24 hours. Acticoat dressings were found to exert maximal bactericidal activity, achieving >10 000 c.f.u./ml reduction of *Staphylococcus aureus* within 24 hours However, the bactericidal activities of the other dressings against *Staphylococcus aureus* were marginal as Aquacel Ag and PolyMem silver dressings, allowed about 10 c.f.u./ml reduction of *Staphylococcus aureus* whereas Urgotul silver dressings allowed an increase in bacterial growth after 24 hours.

A dressing should have sustained effect due to the leaching of the silver ions to inhibit

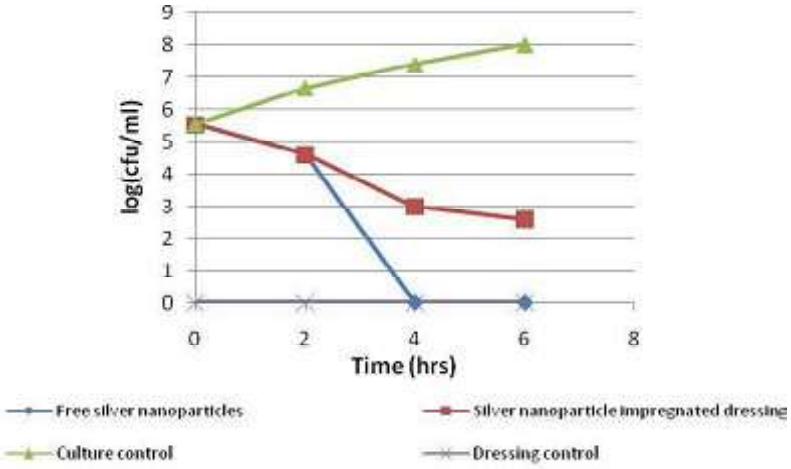

Fig. 5(d). Effect of free Ag nanoparticles and silver impregnated dressings on *Pseudomonas aeruginosa*

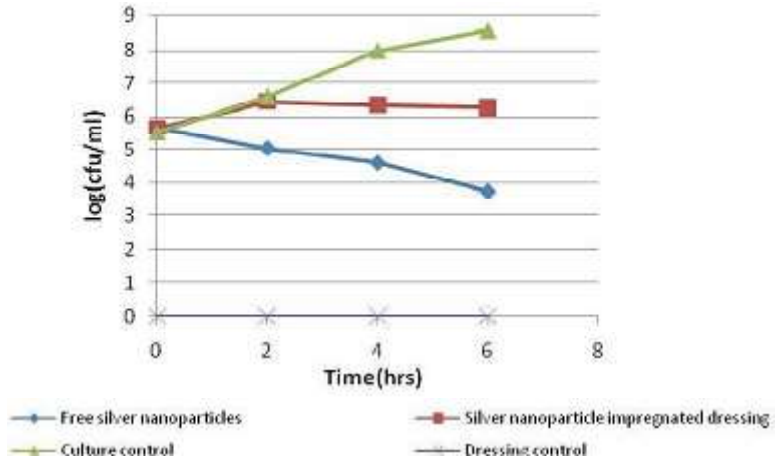

Fig. 5(e). Effect of free Ag nanoparticles and silver impregnated dressings on *Sthaphylococcus aureus*

bacterial growth in the wound exudates in the clinical setting. The concentration and rate of 'bioavailable' silver ions that are released from the surface of the dressing to the wound exudates thus are important factors. SilvaSorb dressings have been used such that it maintained a steady state of 1-2 ppm of ionic silver in isotonic fluid of the wound environment whereas other bandage/ dressing have 546-7.2mg/100cm^2 of silver [17]. Since in our study 3.4mg/100cm^2 of silver nanoparticles has been used, a further increase in the concentration of silver nanoparticle in dressing.

Local staining by silver dressings though a problem it is not a major complication and is usually temporary due to sustained release and high bioavailability, which is furnished by many of the new dressings.[18] Improved methods for silver nanoparticle impregnation along with increased concentration of silver nanoparticle could give a better dressing with increased bactericidal activity especially against gram positive microorganisms

ACKNOWLEDGMENTS

The authors gratefully acknowledge the kindly assistance of Dr.Sujatha Jayaraman, Mr. M.J.Pillai, Dr. Gohil and Mr. Paresh Goti for Scanning electron microscope, Atomic Absorption spectrometer measurements and providing with hospital cultures and bandage materials respectively.

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